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PRINCIPAL INVESTIGATOR: Dr. Brenda Ogle

CONTRACTING ORGANIZATION: University of Minnesota, Twin Cities
Minneapolis, MN 55455-2009

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Brenda Ogle, Caroline Alexander, Ty Harkness
Betty Diamond

E-Mail: ogle@umn.edu

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14. ABSTRACT

The goal of this proposal is to determine whether cell fusion between tumor cells and hematopoietic cells is the precipitating event for breast cancer metastasis and whether viral fusion proteins enable or catalyze this event. If successful, this discovery would dramatically change our approach to breast cancer therapy in the following specific ways. To date we have completed a significant portion of the tasks delineated in Aims 1 and 2. First we have optimized protocols for the separation of myeloid and monocyte populations from human mononuclear cell populations. Last cycle we optimized electroporation conditions for T47D and human mesenchymal stem cell populations and this cycle we have improved our transfection caliber by creating and optimizing lentiviral constructs for delivery of our fusion reporter (BiFC pairs). As a result we have been able to complete our co-culture experiments to determine whether human breast cancer cells fuse spontaneously with hematopoietic cell types. Preliminary results suggest these populations do fuse spontaneously and that fusion products formed in this way can survive several days and are capable of proliferation. Unfortunately, for some cell types our original BiFC pairs did not allow proliferation and so this cycle we reengineered the pair to allow for proliferation of all cell types. Given this unanticipated problem, we have requested an extension and believe we can complete the aims of the grant in this period. In particular, we will purify spontaneous fusion products via flow cytometry and assess for the capacity to migrate and proliferate in vitro. Also, given our compelling early findings, we will investigate the frequency of spontaneous fusion in vivo and be able to discern whether fusion enables breast cancer metastasis.

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INTRODUCTION

Ninety percent of breast cancer-related deaths are due to secondary tumors, or metastases, that form at sites far removed from the primary tumor. Exactly how metastases form is yet unclear. The goal of this proposal is to determine whether cell fusion between tumor cells and hematopoietic cells is the precipitating event for breast cancer metastasis and whether viral fusion proteins enable or catalyze this event. If successful, this discovery would dramatically change our approach to breast cancer therapy in the following specific ways. First, the studies proposed here will help identify fusion partner(s) able to fuse with breast cancer cells to promote a metastatic phenotype. With this information, it may be possible to design strategies to limit interaction with breast cancer cells, including removal of the cell type. In addition, future studies could identify the specific receptor-ligand interactions necessary for cell fusion, to produce a target for drug therapy. Post-fusion events might also be investigated, including the molecular steps governing the integration or rearrangement of genomic DNA to form a single hybrid genome or those steps necessary for activation of genes that regulate the migratory or invasive phenotype. Second, the studies proposed here will investigate the possibility that exogenous, virus-associated proteins might facilitate breast cancer cell fusion. If viral fusogens are found to promote tumor cell fusion, viral vaccination regimes may be appropriate as a prevention strategy. Vaccines might be developed to target viral fusion genes (i.e., fusogens) exclusively, so that the immune system would recognize the protein, even in the context of a eukaryotic cell membrane. Third, the studies proposed here will establish new tools for the study of the complex processes of cell fusion. The inducible bipartite nature of these strategies assures the accurate identification of fusion products, and allows for longitudinal assays both *in vitro* and *in vivo.*

BODY

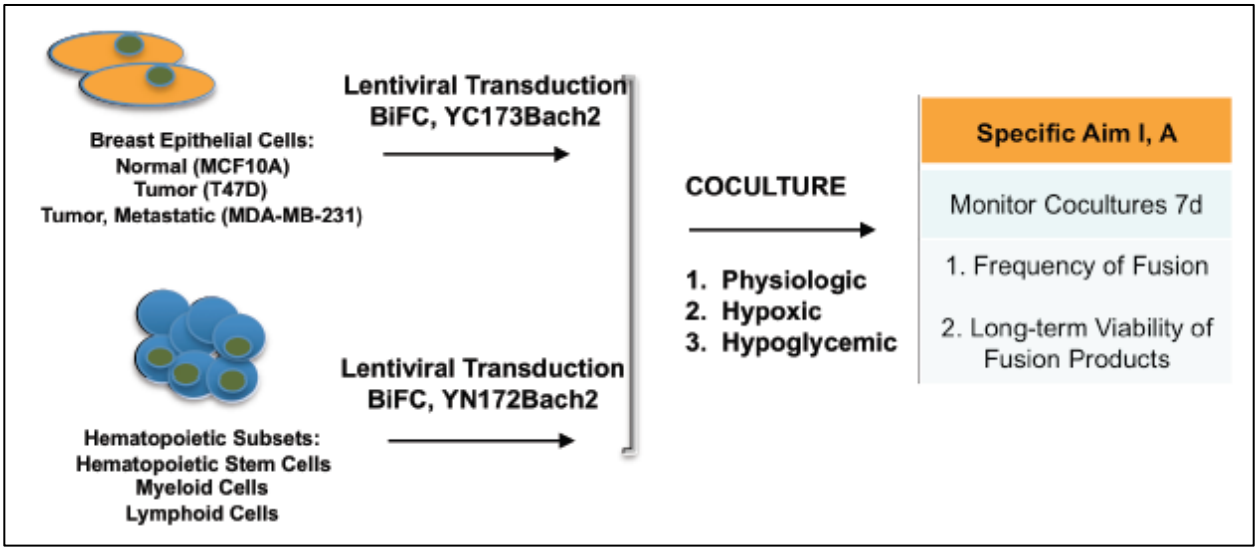
The research accomplishments made to date relate to both Specific Aim 1 and 2. Here we reiterate the aims and describe progress on tasks related to the aims as they were delineated in the original statement of work. Of note, the majority of the tasks of Aim 1a are complete and were reported last period. And excitingly, we found that breast cancer cells (and normal breast epithelial cells) can fuse spontaneously with hematopoietic cell types. Unfortunately, at the beginning of this reporting period, we also unexpectedly discovered that some fusion partners were unable to proliferate after transfection with the vectors containing BiFC counterparts (our inducible reporter of cell fusion), and subsequently underwent cell death. We have therefore spent a portion of this period engineering a new BiFC system that does not inhibit proliferation and thereby enables long-term cell health.

Specific Aim 1. To determine the specificity and functional capacity of hematopoietic cells that spontaneously fuse with breast cancer cells in vitro.

Aim 1b. Determine the migratory and invasive capacity of blood cell-tumor cell fusion products.

Task 4 - Prepare breast cancer cell-hematopoietic subpopulation co-cultures

Normal breast epithelial cells or breast cancer cell lines were transfected with one half of the BiFC system via lentiviral transfection. Hematopoietic cell types were transfected with the other half of the BiFC system via lentiviral transfection and the cell types were cocultured according to the following schematic.



Of great relevance, we found that normal human breast epithelium can fuse spontaneously with human mesenchymal stem cells at a frequency 0.003% ± 0.003%. Similarly, highly metastatic breast tumor line, MDA-MB-231 can also fuse spontaneously with human mesenchymal stem cells at a frequency somewhat higher than that of normal epithelium (0.03% ± 0.01%, **Figure 1**). Spontaneous fusion occurred under physiologic conditions and we are now working to optimize hypoxic and hypoglycemic culture conditions to determine whether metabolic stress of this type can further stimulate spontaneous fusion.

Unanticipated challenges of the BiFC plasmids

The BiFC probes were proposed here as an inducible means to detect fusion in vitro. The BiFC probes are coupled to histone H3.1 as a means to bring them in close proximity in a fusion product. However, we have found that certain cell types are particularly sensitive to the coupling a fluorescent molecule to that region of the histone. In this case, the cell or fusion product attempts to initiate proliferation but cannot do so successfully and the result is cell death (**Figure 2**). Particularly sensitive to BiFC transfection were MSCs, MCF10A and MDA-MB-231 cells. Despite this problem, we have been able to determine whether spontaneous fusion occurs (Aim 1a), but not the long term functional outcomes of fusion (Aim 1b). Therefore, we have attempted multiple strategies to circumvent this limitation. The most successful is the deletion of Histone H3.1 coupling from one half of the BiFC probe set. In this way, BiFC still couples in the nucleus, but irreversible binding does not involve linking of histones and instead just the fluorescent moieties. With this approach proliferation appears possible and cell viability long term is improved (**Figure 3**). Extensive studies and complete quantification of the new vectors and their impact on viability is near completion.

Specific Aim 2. To determine whether tumor cell fusion products give rise to metastatic tumors.

While optimizing the BiFC plasmids above, and given the promising evidence of spontaneous fusion of breast epithelium with MSCs, we began to prepare for *in vivo* studies. Thus far we have completed the following tasks associated with aims 2a and 2b.

Aim 2a. Track the course of fusion products with tumor metastasis/sensitivity of detection

Task 1. Inject luciferase expression cells into mice and image; xenogen system (**Figure 4**)

Aim 2b. Track the course of fusion products with tumor metastasis/point of metastasis

Task 1. Breeding of autochthonous mice and *Cre* mice. Breeding of *Cre* mice is successfully underway.

To address the tasks above, MSCs expressing viral fusogens and floxed luciferase were transplanted to the murine myocardium. To determine whether transplanted cells could fuse with the cells of the ventricle and persist for at least a week, live animal imaging for luminescence (and therefore cell fusion) was conducted eight days after transplantation. A distinct luminescent signal was detected in the excised heart of all (n = 4) mice receiving transplanted cells ((1.37 $\pm$ 0.24), intensity when normalized to luminescence of excised negative control heart which did not receive any hMSCs. $P < 0.01$ when compared to luminescent intensity in non luminescent organs (ie. kideny)) **Figure 4**). Interestingly, luminescence was also detected in the stomach and small intestine of transplanted mice, at levels similar to that observed in the heart. The stomach had the highest intensity (1.53 $\pm$ 0.51) while, the small intestine intensity was slightly lower (1.44 $\pm$ 0.16), (n=4 mice, signal normalized to luminescence of corresponding excised negative control organs of mice that did not receive hMSCs, $P < 0.01$ when compared to luminescent intensity in non luminescent organs (ie. kideny)). Some of the mice receiving cell transplants also exhibited low levels of luminescence in the liver (1.14 $\pm$ 0.06) (n=4, $P < 0.05$ when compared to luminescent intensity in non luminescent organs (ie. kideny)). Other distal organs exhibited no luminescence (i.e. kidneys 0.98 $\pm$ 0.03). The detected luciferase activity is a reflection of the number of cells expressing luciferase and therefore a reflection of the relative number of cell fusion events. These results demonstrate that fusion has occurred at the delivery site and in distal organs in the cre mice.

KEY RESEARCH ACCOMPLISHMENTS

- Optimization of MACS magnetic bead separation of myeloid and monocyte populations from human buffy coat (previous report)
- Optimization of electroporation transduction conditions for T47D cells (previous report)
- Optimization of lentiviral constructs for BiFC transfection (previous report)
- Engineering of new BiFC pairs to promote viability and proliferative capacity of all cell types of interest for this proposal (this report)
- Indication of spontaneous fusion between MCF10A, MDA-MD-231 and hMSCs and between T47D and hMSCs in *in vitro* cocultures (this report)
- Fate of putative T47DxhMSC fusion products tracked over time; some fusion products were found to undergo proliferation (previous report)
- The course of fusion products after transplantation of MSCs expressing the floxed luciferase to cre mice was tracked. (this report)

REPORTABLE OUTCOMES

Manuscript:
Harkness, TE; Weaver, BA; Alexander, CM; Ogle, BM. Cell Fusion in Tumor Development: Accelerated Genetic Evolution. *Critical Reviews in Oncogenesis*. 18(1-2):19-42. 2013.

Degrees awarded:
Ty Harkness, M.S., University of Wisconsin-Madison.

Employment received: Ty Harkness, Research Scientist, Pfizer, San Francisco, CA

Research tools developed: Bimolecular fluorescence complementation lentiviral vectors for study of cell fusion. Specifically, VN-H3.1, YC-H3.1 and VN-null.

CONCLUSIONS AND FUTURE STUDIES

In the upcoming months we expect to utilize the new BiFC construct in lentiviral format to transfect multiple types of blood cells, healthy mammary epithelial cells, and transformed mammary epithelial cells of varying degrees of metastatic capacity. We will work with both human and murine cells in parallel. Combinatorial cocultures with different blood cell subpopulations (myeloid, lymphoid, and monocyte) and healthy or diseased mammary lines will be performed to determine the frequency of fusion between these subpopulations. The effect of hypoxia and hypoglycemia on the frequency of fusion will also be determined. Identified fusion products will then be purified via flow cytometry and assessed by migration and proliferation studies.

In vivo studies will also be initiated using the Cre-Lox luciferase system in murine models of breast cancer. If possible, blood cell fusion partners identified in *in vitro* studies will be selectively transduced with the Cre gene to specify *in vivo* breast cancer fusion products.

SUPPORTING DATA

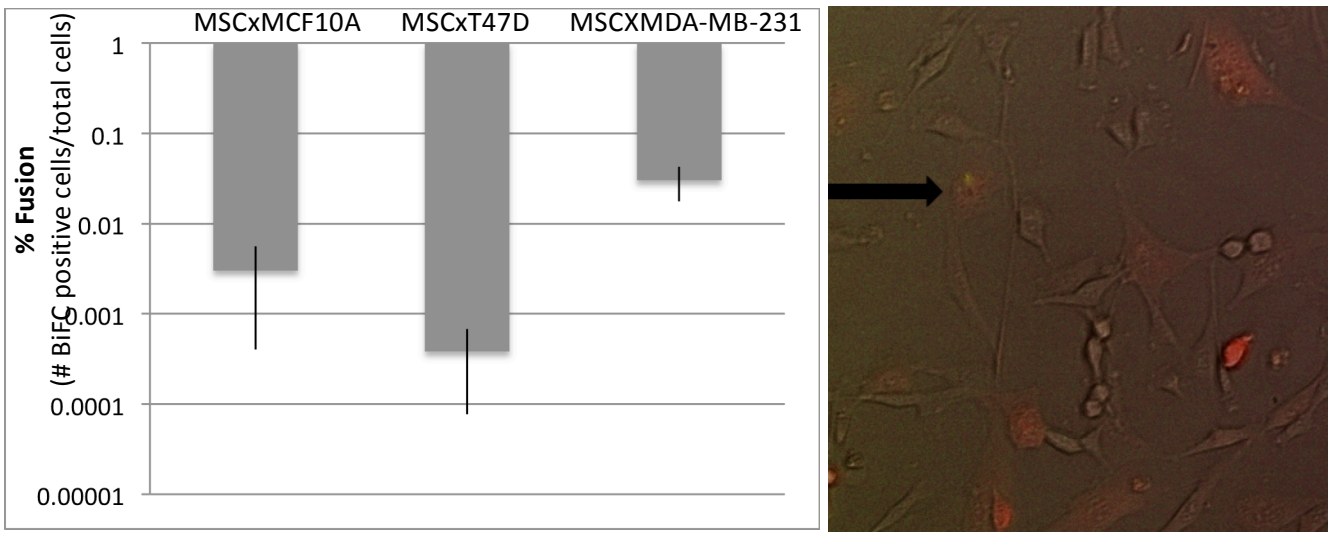


Figure 1. Spontaneous fusion of human mesenchymal stem cells with either human breast epithelium (MCF10A), weakly metastatic breast tumor line (T47D) or highly metastatic breast tumor line (MDA-MB-231). Mesenchymal stem cells were transfected with BiFC half VN-H3.1 and breast epithelium or breast tumor lines were transfected with BiFC half YC-H3.1 via lentivirus. Twenty four hours after transfection, cocultures were initiated and cultures were monitored for fluorescent signal indicative of cell fusion. The number of fluorescent cells was determined for each coculture and reported as a percentage of the total number of cells per well. n = 6 wells from 3 separate experiments. Error bars = standard deviation. Image to the right shows a fusion product (black arrow) formed between MCF10A and MSC. Note green fluorescence signal in nucleus indicative of BiFC pairing and therefore cell fusion. Red fluorescence indicates cells that have received one half of the BiFC pair.

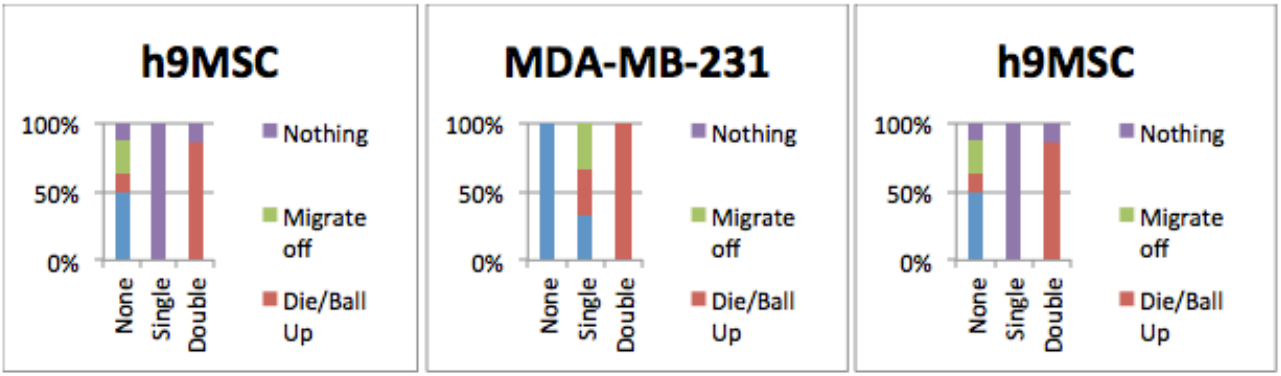


Figure 2. Cell viability following double transfection with the BiFC plamids. To determine whether the presence of both BiFC plasmid in the same cell (akin to the scenario for fusion products would decrease long term cell viability, time lapse microscopy was used to track fluorescently labeled cells over 48 hours. The lines displayed above were particularly sensitive to the BiFC protein product and suffered death by 48 hours. Thus the BiFC plasmids in their current form were used to detect the frequency of spontaneous fusion only and efforts were initiated to improve the BiFC strategy to enable efficient proliferation.

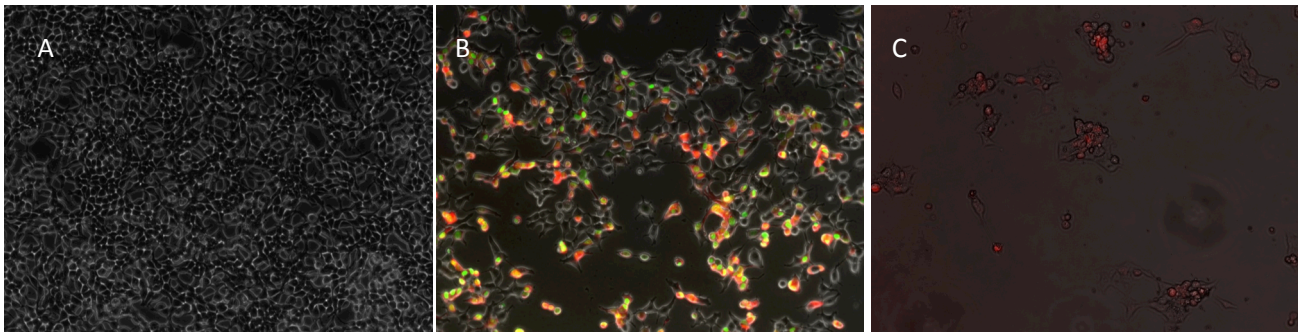


Figure 3. Viability of MDA-MB-231 cells following transfection with BiFC plasmids. A, Untransfected MDA-MB-231 cells. B, MDA-MB-231 cells transfected with modified VN-YC BiFC pairing. Red indicates successful transfection with one BiFC half. Green indicated successful transfection of both BiFC halves (simulating a fusion product) C, MDA-MB-231 cells transfected with original VN-YC BiFC pairing. Red indicates successful transfection with one BiFC half. Green indicated successful transfection of both BiFC halves (simulating a fusion product). Note limited cell survival and no evidence of survival of cells containing both BiFC halves.

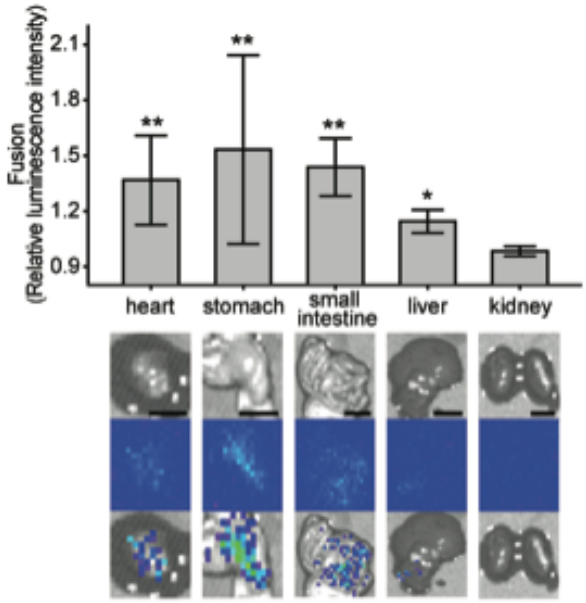


Figure 4: Detection of fusion at delivery site and distal organs in cre mice. Relative luminescent intensity from heart, stomach, small intestine, liver and kidney (left to right), normalized to the luminescence intensity of the same organ in a mouse that did not receive MSCs (n=4 mice). Luminescent signal from heart, stomach, small intestine and liver was significantly higher than that of corresponding control organs and kidney tissue of treated mice (** $p < 0.01$, * $p < 0.05$). Representative images for each organ are shown beneath the graph. From top to bottom: photograph, luminescence intensity, overlay.

Cell Fusion In Tumor Development: Accelerated Genetic Evolution

Ty Harkness[*], Beth A. Weaver[π,+], Caroline Alexander[+, ¶] and Brenda M. Ogle[*, ¶, §]

Departments of Biomedical Engineering[*], Cell and Regenerative Biology[π], the Carbone Cancer Center[+], the Laboratory for Optical and Computational Instrumentation[¶], the Cardiovascular Physiology Core Facility[#], and
the Material Sciences Program[§]
University of Wisconsin at Madison, Madison, Wisconsin 53706, USA

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Address correspondence to:
Brenda Ogle
University of Wisconsin-Madison
2-114 Engineering Centers Building
1550 Engineering Drive
Madison, WI 53706
Phone: 608-265-8267
ogle@wisc.edu

Abstract

The majority of human tumor cells have highly aberrant karyotypes, typically ascribed to errors during tumor cell division, potentially linked to a failure of DNA repair, or telomeric insufficiency. Here we discuss another option, that of cell fusion, which can lead to the re-assortment of chromosomes during post-fusion mitosis. The observation of hyperdiploid cells has a long history in cancer genetics, but the concept of cell fusion has been difficult to test in practice. Here, we examine the role of cell fusion during normal development, and relate that to potential cellular fusion partners for primary tumor cells. In particular, we describe the potential for stromal partner fusion during metastatic mobilization. The evidence for genetic and cytoplasmic diversity in heterotypic fusion partners is described, together with the new tools available to help the evaluation of this process as a tumor driver.

Introduction

Cell fusion is an incompletely understood process that occurs spontaneously during normal development as well as in response to viral infection, including infections known to promote transformation, such as HPV.[1] By increasing DNA content as well as centrosome number, cell fusion rapidly alters cellular genotypes and phenotypes and catalyzes genetic diversity. Through stimulating genetic diversity, cell fusion may contribute to the formation, propagation and metastasis of tumor cells. For instance, fusion of a healthy epithelial cell with an activated fibroblast of the local stroma can allow it to acquire the ability to proliferate inappropriately. Fusion of a tumor cell with a mesenchymal stem cell can allow it to degrade extracellular matrix proteins and migrate beyond the basement membrane. Fusion of an epithelial tumor cell with a macrophage could lead to the temporary acquisition of macrophage-associated properties of transit through the vasculature and invasion of distant tissue sites.

The current view of cancer is evolving beyond the accumulation of genomic and epigenetic aberrations in epithelial cells to one that appreciates the impact of the "soil"[2] or microenvironment on the formation, propagation and metastasis of tumor cells. Focus of this effort centers on the impact of close contact with cellular members of the stroma and immune system, which generate insoluble extracellular matrix proteins and/or soluble paracrine and autocrine factors. Activated stromal fibroblasts secrete a myriad of soluble factors including SDF-1,[3] MMPs,[4] VEGF-A,[5] HGF,[6] Sdc1[7] and TGF-β[8] that have been shown to enhance tumor growth and angiogenesis. As one example, cells of the human breast cell line MCF-7 are weakly tumorigenic in SCID mice, but are strongly tumorigenic when inoculated with stromal fibroblasts.[9-10] In addition to secreted factors, the impact of cell-cell communication is also coming to the fore as a means by which the microenvironment facilitates oncogenesis. Recent

work by Apostolopoulou et al shows that MCF-7 breast cancer epithelial cells form cadherin-23-dependent, heterotypic adhesions with normal breast fibroblasts when grown in co-culture, and suggests that cadherin-23 upregulation may play a role in the early stages of metastasis.[11] A logical extension of cell adhesion or close cell contact is the possibility that tumor cells or their precursors fuse with cells of the local microenvironment. Known examples of cell fusion, along with their demonstrated and potential roles in oncogenesis and metastasis are reviewed here.

II. CONTEXT AND OUTCOMES OF CELL FUSION

A. Spontaneous Heterotypic Fusion Occurs in Nature

Fusion occurs spontaneously between cells of the same type (homotypic) and cells of different types (heterotypic). Homotypic cell fusion was first observed more than eighty years ago in the formation of foreign body giant cells[12] and was soon followed by reports of fusion between trophoblasts of the placenta, myoblasts of muscle fibers and osteoclasts of bone.[13] However, it was not appreciated until recently that fusion products may form between heterogeneous cell types *in vivo* and that nuclei themselves often fuse to give rise to synkaryons.[14] Spontaneous heterotypic cell fusion *in vivo* was first observed in transplantation studies in animal models and later in humans, both in healthy and diseased states.[15-18] Mesenchymal stem cells and other bone marrow-derived cells (BMDCs) have often been implicated in these studies, especially in synkaryon formation.[19-20]

B. Cell Fusion Generates Diversity

The immediate consequence of both homotypic and heterotypic cell fusion is tetraploidy, a doubling of the genetic material available to the cell and the first opportunity to augment clonal

diversity. Given the correlation between copy number and gene expression, tetraploidy alone has the potential to radically alter cell state.[21-22] Tetraploidy has also been reported to increase the sensitivity of both yeast and human cells to DNA-damaging agents.[23-24] Fusion between cells in different stages of the cell cycle can also result in DNA damage – particularly double-strand breaks mediated by premature chromosome condensation.[25-26] In addition, tetraploidy has been suggested to "overwhelm" the mitotic machinery, making chromosome missegregation more likely[25]. A second immediate consequence of cell fusion is an increase in centrosome number. Supernumerary centrosomes can result in multipolar spindles or the missegregation of individual chromosomes after multipolar spindles are focused into bipolar ones.[27-28] In any scenario, fusion would be expected to increase genetic diversity of the fusion product compared to individual fusion partners.

Of course genetic diversity is likely to result in phenotypic diversity, dependent on the degree of change and the localization of change. Perhaps the most familiar example of how a fusion product attains diversity of phenotype is the hybridoma cell, a Sendai virus-induced fusion of a murine myeloma cell with a B cell from an immunized mouse.[29] The entire hybridoma system is based on the assumption that the fusion product has a competitive advantage over the parental cells, as neither parental cell line can survive in selective media. The fusion products of these experiments were known to have highly variable phenotypes. In practice, this made the process of hybridoma selection frustrating, since only a percentage of fusions resulted in nuclear hybrids, a fraction of those produced antibodies, and a small percentage of antibody-producing hybrids were able to be expanded into functional hybridomas.[30] The ability of hybridomas to "evolve" upon continuous culture was even used to map genes to specific chromosomes.[31] In a study that mapped an "IL-6 dependency gene" to human chromosome 21 it was found that newly

fused hybridomas displayed IL-6 dependence, however this dependency was lost at frequencies up to 50% upon continuous culture, suggesting that genetic material was lost after fusion.[31] Karyotyping of stable clones showed a great variety of seemingly randomized chromosome content. Thus, early hybridomas show a high degree of chromosomal instability (CIN), defined here as an increased and continuous rate of large chromosomal aberrations, including deletions, duplications, or translocations as well as loss or gain of whole chromosomes. Despite the great degree of genetic rearrangement after fusion, hybridoma genotypes eventually stabilize in culture, as evidenced by the extent of their use in industry to provide an extremely pure monoclonal antibody product in a highly reproducible fashion.[32-33]

Although the progression from tetraploidy to aneuploidy or diploidy in hybridomas may be dismissed as a product of extreme culture conditions, there is considerable evidence that this process also occurs in normal culture conditions and even *in vivo*. Observations that tetraploid yeast cells could progress to aneuploidy,[34] and that cells with more than four centrioles could be found in mouse models,[35] along with the fact that multipolar mitoses are often seen in human cancers[36] led to the hypothesis that supernumerary centrosomes lead to multipolar cell division in tetraploid cells and diverse aneuploid progeny.[36-37] However, multipolar spindles are frequently focused into bipolar spindles before anaphase onset and chromosome segregation.[28] Ganem et al. recently showed that, even when cells ultimately proceed through a bipolar division, extra centrosomes promote missegregation of individual chromosomes in a variety of human cell lines by merotelic attachment, in which a single kinetochore attaches to microtubules originating from multiple centrosomes.[27] If these centrosomes are focused into different spindle poles, this phenomenon can lead to chromosome lagging during anaphase and the production of diverse aneuploid progeny even in the absence of multipolar division.

Diversification of the genome and the phenome could be advantageous in many scenarios beyond the generation of hybridomas. As one example, Grompe and colleagues have reported detailed studies of the consequences of hepatocyte fusion with BMDCs in a regenerative context *in vivo*. It was first shown that hepatocyte-BMDC fusion products had a competitive advantage in the diseased liver microenvironment and were able to completely repopulate the host liver.[38-39] After diploid fusion products (as determined by a Cre-Lox reporter system) were observed[19] genetic studies were performed to elucidate the mechanism of chromosome loss after cell fusion. Using a three-marker system in which fusion-derived hepatocytes alone expressed β-gal, FAH, and bore a Y-chromosome, ploidy reduction was demonstrated, frequently resulting in aneuploid progeny of diverse karyotypes.[40] Subsequent single-cell PCR genotyping revealed that parent-specific markers segregated independently via ploidy reduction after cell fusion, with the incidence of loss ranging from 33-70% of cells analyzed. Only 13% of fusion products tested retained all parental markers, suggesting that ploidy reduction is a very common phenomenon after cell fusion *in vivo*. It is also interesting that the incidence of single marker loss did not cluster around 50%, as would be expected for random chromosome loss, again suggesting that environmental pressure may enrich for cells with favorable phenotypes. Later studies also implicated merotely and chromosome lagging in aneuploidy generation in this process.[41] In addition, it was shown that hepatocytes can regularly increase and reduce their ploidy while maintaining viability and producing high levels of genetic heterogeneity – a process termed the "ploidy conveyor".[41] It is interesting to note that regrowth of the liver after hepatectomy is associated with an increase in polyploid cells.[42] Populations of tetraploid fibroblasts have also been observed in a variety of wound healing contexts in humans and mice and the frequency of tetraploidy has been shown to increase during the proliferative phase of wound healing.[43]

Importantly, these tetraploid cells often appeared in clusters suggesting a clonal evolution, possibly from an initial cell-cell fusion event. It is easy to envision an invading inflammatory BMDC being involved in this context as well. These examples suggest that polyploidy may in fact be an adaptive response to the need to repopulate a damaged tissue.[44]

While genetic recombination is certainly an important event in heterotypic fusion, the mixing of epigenetic factors from diverse cell types may be just as crucial. In a process termed "nuclear reprogramming",[45-46] cell fusion can result in activation or silencing of genes of one parent via outside epigenetic factors. The most well-known example of nuclear reprogramming by the cytoplasm is probably somatic cell nuclear transfer, which has been utilized to reset a mature nucleus to a pluripotent or totipotent state and resulted in the cloning of Dolly the sheep.[47] Decades ago, it was shown that trans-acting epigenetic factors from one nucleus of a heterokaryon can silence or activate genes in a partner nucleus.[48-49] Through work in stem cell biology, it has been shown that fusion of pluripotent murine germ cells with thymocytes from adult mice alters the methylation status of previously imprinted somatic genes.[50] However, when embryonic stem cells were used as fusion partners instead of germ cells, imprinted genes were not demethylated but hyperacetylation of histones H3 and H4 led to a pluripotent state.[51-52] The result of these epigenetic changes can be expression of genes already active in one (or both) fusion partners[48] or expression of genes not expressed in either parent, an outcome seen more recently in the context of cancer.[53]

An altered phenotype may also be produced solely by the physical changes of a fusion product, specifically an increase in cytoplasmic and/or nuclear volume. Effects of increased volume could include less efficient transfer or decreased concentration of transcription factors and other proteins, less efficient interactions between organelles, and lowered efficiency of

membrane-dependent activities due to an increased ratio of cell volume to organelle surface area.[44]

The process of combining genetic or phenotypic information to create diversity and selective competitive advantages is also an important concept in evolutionary biology. There is considerable evidence of polyploidization in the evolutionary history of many organisms including mammals,[54] which although not well understood is thought to increase evolutionary innovation through genome recombination.[55] Furthermore, a recent study on a newly formed (~80 yr) naturally polyploid plant species revealed massive chromosomal instability, with 69% of individuals studied displaying aneuploidy for one or more chromosomes and 76% showing intergenomic translocations.[56] The high degree of genomic diversity still being generated after ~40 generations of this biennial species suggests that prolonged periods of chromosomal instability may commonly precede genomic stabilization in naturally forming polyploid species. The evolutionary theory of symbiogenesis takes this idea a step further.[57] It holds that mergers of species, rather than Darwinian evolution via accumulation of mutations, are the drivers of diversity and speciation. The most convincing evidence to support the symbiogenic theory is the finding that mitochondria and chloroplasts are of bacterial origin.[58] What fraction of evolutionary adaptations can be ascribed to symbiogenesis is unknown, but it is not difficult to imagine a corollary to symbiogenesis, which states that one means by which species or structures of species are merged is via fusion.

III. MECHANISMS OF CELL FUSION

A. Spontaneous Cell Fusion

Cell membranes allow the cell to function independently and preserve self-regulation from outside influence. In normal development, this compartmentalization is overcome by cell-cell fusion only in a tightly regulated manner. The process of fusion is thermodynamically unfavorable owing to the need to expunge water between the cells, the negative and opposing charges of the outer phospholipid bilayer, and the need to disrupt the membrane. One way that the formation of a syncytium is enabled is via specific integral membrane proteins, termed fusogens, which minimize the incredible energy cost required to overcome the merger of the two hydrophobic membranes.[59] Establishing true fusogens has proven difficult since removal of facilitating proteins from the system reduces overall fusion rates. As technologies advance, many "fusogens" have been contested and shown to be merely adhesion proteins that bring cell membranes in close apposition but do not actually facilitate fusion. Although the mechanisms of spontaneous cell fusion are still not well understood, studies of fusion in normal development have given us some clues.

B. Homotypic Fusion: Macrophages

While some cells, such as myoblasts and placental trophoblasts, are required to fuse for normal development, macrophages are normally present as mononuclear cells in the body, and undergo rare (and inducible) fusion events to form multinucleated osteoclasts and giant cells.[60] As such, macrophages may prove to be the most useful model to study inducible fusion. When the recent evidence of BMDC fusion with somatic and cancer cells is taken into account, the mechanisms of macrophage fusion become even more interesting.

Osteoclast and giant cell development is thought to progress similarly and include a series of functional steps including induction of a fusion-competent status, chemotaxis, cell-cell

attachment, cytoskeletal rearrangements, and fusion.[61-62] IL-4 and IL-13 are secreted by a number of immune cells and can induce giant cell formation *in vitro* and *in vivo*,[61] while RANKL and M-CSF can induce osteoclast formation *in vitro*.[63] These soluble factors are thought to lead to a fusion competent status by upregulating fusion mediator expression on macrophage cell membranes. For example, IL-4 stimulates DC-STAMP and E-cadherin expression, both of which are implicated in macrophage fusion.[64-66] Internal signaling via DAP12 also seems to be important in this process by mediating transcription of other fusion mediators.[67] Little is known about chemotactic and adhesive factors in macrophage fusion. CCL2 (MCP1) is thought to be important in chemotaxis, as deficiency in mice has been shown to impair giant cell formation while leaving macrophage recruitment unaffected,[68] and E-cadherin has been implicated in macrophage adhesion.[61] Final temporal regulation of macrophage fusion may be determined by the macrophage fusion receptor (MFR) and its ligand CD47. CD47 is expressed ubiquitously, while MFR is expressed only in myeloid cells and neurons. Additionally, MFR is strongly and transiently expressed in macrophages at the onset of fusion.[69] Notably, CD47[70-71] and CCL2[72-73] are both expressed in diverse cancers[74] and IL-4 and IL-13 are secreted by $CD4^+$ T cells in the breast tumor microenvironment.[75]

It has recently been shown that a podosome-like structure is created around actin foci in fusion-competent myoblasts. These podosomes invade adjacent founder myoblasts and mediate fusion pore formation.[76] Interestingly, lamellipodia form in IL-4 stimulated macrophages before giant cell formation, and these structures have been suggested to be critical in the fusion process.[77]

C. Heterotypic Fusion: Gametes

Despite its essential role in the generation of life, the most well known example of developmental heterotypic cell fusion is similarly shrouded in mystery. Mammalian reproduction occurs through heterotypic gamete fusion. As a fusion product, the fertilized embryo can proliferate and differentiate into all the tissues of the adult body as well as the extra-embryonic tissues. CD9 is an egg-associated putative fusogen. Knockout mice for CD9 have show severely hampered fertilization[78] which is restored with polyadenylated CD9 mRNA.[79] Additionally, CD9 has been shown to generate the strongest observed interactions with the sperm.[80] In contrast, Izumo is a putative fusogen found only on the sperm membrane following acrosomal reaction.[81] Supporting the role of Izumo as a fusogen is the finding that Izumo-deficient mice have normal sperm migration into the oviduct, motility, zona pellucida penetration, and acrosomal reaction, but are completely insterile.[82] Furthermore, successful fertilization occurs after artificial injection of Izumo-deficient sperm into oocytes, indicating that Izumo-null mice lacked other developmental defects. Although the expression of each protein is essential for fusion and fertilization, the molecular mechanism of Izumo/CD9 induced fusion is not well understood. For example, it is not known whether other facilitating proteins are required.[59, 82]

D. Viral-Mediated Fusion

In contrast to spontaneous cell-cell fusion, mechanisms of many virus-cell fusion events have been well characterized.[83] The first step of viral fusion is attachment; capsid proteins bind to specific receptor proteins on the cell membrane. Next, depending upon the type of virus, one of two pathways occurs: plasma membrane fusion or endocytosis followed by endosomal membrane fusion. Typically, a hidden fusogenic protein in the envelope is activated either by the

induction of a conformational change upon receptor binding, or exposure to low pH within the endosome. Class I fusion proteins, such as the human immunodeficiency virus type-1 (HIV-1) envelope protein (Env) use a hydrophobic fusion peptide that is only exposed after specific receptor binding or under low pH conditions. The fusion protein then undergoes a conformational change that brings the viral and cell membranes into close apposition to mediate fusion.[84]

After viral infection, cells may express fusogenic proteins such as Env on their cell membranes, facilitating fusion with adjacent healthy cells. This characteristic has been used to induce cell fusion *in vitro* for years[85] and viruses that are able to fuse cells are nearly ubiquitous in humans.[26] This property is especially interesting in the context of oncogenesis in light of recent findings that certain viruses such as HPV are indeed causative of cancer and capable of inducing cell fusion.[1, 86] It is also worth noting that tumors often create an acidic microenvironment that could activate pH-sensitive viral fusogens.[87]

IV. CELL FUSION AND ONCOGENESIS

CIN and aneuploidy are classic hallmarks of cancer[88-89] and are documented consequences of cell fusion.[25] However, it is unclear exactly how CIN is initiated in cancer progression. Supernumerary centrosomes, with or without tetraploidy, can lead to CIN due to the formation of merotelic attachments.[27, 36] It has also been suggested that CIN could arise as a direct result of extra chromosomes through an increased rate of DNA damage [34, 90] or that increased chromosome content could "overwhelm" the mitotic machinery resulting in missegregation of chromosomes.[25, 37] As was discussed earlier, cell fusion-induced tetraploidy can lead to aneuploid progeny through a transient period of CIN. Fittingly, the first clue that

fusion may be involved in oncogenesis is that tetraploidy is common in premalignant lesions and often gives way to aneuploidy in later stages.[25, 91-92] The best studied example of this phenomenon is probably Barret's esophagus, a premalignant condition in which tetraploid cells are predictive of progression into both aneuploidy and cancer.[93-94] Tetraploidy has also been detected in premalignant lesions in cervical cancer,[95] head and neck squamous cell carcinoma,[96] and Kaposi sarcoma,[97] however it is unknown whether tetraploid cells in these lesions progress to malignancy. Direct evidence for carcinogenesis as a result of tetraploidy comes from a 2005 study in which $p53^{-/-}$ tetraploid cells were derived through chemically inhibiting cytokinesis. [98] These $p53^{-/-}$ tetraploid cells formed tumors when implanted subcutaneously in nude mice while isogenic $p53^{-/-}$ diploid cells did not. Karyotyping of resultant tumors showed they were near-tetraploid with numerous structural rearrangements. The observations that a majority of cancer cell lines in the NCI-60 drug screening panel[99] and elsewhere[100-102] are hyperdiploid and that karyotypes are generally preserved between cell lines and the primary tumors they were derived from[100, 103] suggest that the progression from tetraploidy to aneuploidy may be common in many cancers *in vivo*.

It has been reported that there exists a p53-dependent "tetraploidy checkpoint" that must be overcome for proliferation of these cells.[104-105] However, subsequent experiments using the same cell type have failed to replicate this finding.[24, 106] Even though cell cycle arrest is not directly related to DNA content, suppression of proliferation is common in fusion products, as evidenced by the low survival of hybridomas[30] and lack of proliferation of developmental fusion products such as osteoclasts and muscle fibers.[13] Additional proof was provided by Duelli et al. in the context of viral-induced cell fusion. It was observed that cell fusion induced by viral infection of normal human fibroblasts, but not the viral infection itself, caused cell cycle

arrest.[107] However, if one parental cell expressed the adenoviral oncogene E1A or a mutated form of the tumor suppressor p53 the fusion products proliferated, producing diverse aneuploid progeny, some of which were capable of producing tumors in nude mice.[108] Furthermore, Duelli et al. have suggested that fusion-specific mechanisms of combining DNA content from cells in different epigenetic states, possibly including premature chromosome condensation, may result in instability, double strand breaks, and consequent translocations often seen in aneuploid progeny.[26]

An alternative explanation of the appearance of diverse aneuploid progeny after a period of CIN in cancer progression is progression through "telomere crisis". This theory holds that while a great majority of cells will trigger senescence or apoptosis pathways upon sufficient erosion of telomeres (i.e. reaching the Hayflick limit), oncogene expression may allow continued proliferation to the point of crisis. Telomere crisis is characterized by genetic aberrations including chromosomal end-to-end fusions, translocations, losses, and duplications. Out of this period of genetic instability, rare cells emerge that are able to maintain telomere length by reactivating telomerase. These dysregulated aneuploid cells may then go on to form malignant tumors.[109-110]

In a landmark study of *in situ* genome instability in breast cancer, Chin et al. demonstrated a transient period of genomic instability coinciding with telomerase activation and with transition from ductal hyperplasia to ductal carcinoma *in situ*.[110] This period of instability was attributed to telomere crisis, although telomere length was also shown to decrease at a steady rate throughout disease progression. However, two observations made by Chin and colleagues are reminiscent of CIN following cell fusion. First, it was noted that the increase in genome instability was contemporaneous to an increase in DNA content. Second, the frequency of

anaphase bridges were low in hyperplasia, highest during the period of instability, and reduced thereafter. The occurrence of anaphase bridges is often used as an indicator of telomere crisis,[111-112] but can also occur as a consequence of chromosome lagging due to supernumerary centrosomes and merotely as discussed earlier.[27, 113] Thus, the same temporal pattern of anaphase bridge frequency peaking alongside increased DNA content and genomic instability would be expected to occur after cell fusion.

Further confusing the debate around CIN and aneuploidy in the context of cancer is that aneuploidy can act as a tumor promoter or suppressor depending on the cellular context.[114] Aneuploidy caused by depletion of the mitotic checkpoint component BubR1 promotes tumor formation in the colon of $APC^{Min/+}$ mice, which are predisposed to intestinal tumors due to heterozygous expression of a truncation mutant of the Adenomatous Polyposis Coli tumor suppressor, but inhibits tumor formation in the small intestine.[115] Similarly, monosomy of 33 genes on mouse chromosome 16 enhances intestinal tumor formation in $APC^{Min/+}$ mice, but trisomy of these same 33 genes suppresses tumor formation.[116] Aneuploidy caused by partial reduction of the mitotic checkpoint component Bub1 drives liver tumor formation, but further reducing the level of Bub1 causes an increased rate of chromosome missegregation and suppresses liver tumors.[117] Both loss and overexpression of securin, a protein that prevents premature sister chromatid separation, result in aneuploidy.[118-119] However, loss of securin reduces pituitary tumor formation in $Rb^{+/-}$ animals,[120] while overexpression of securin facilitates pituitary tumor development.[121] Finally, epidemiological studies have shown that Down's syndrome/trisomy 21 patients have much higher rates of hematopoietic malignancies, but lower incidence of solid tumors.[122-124]

In another study, Weaver et al. utilized a low-expression Centromere-associated Protein-E (CENP-$E^{+/-}$) model to induce aneuploidy and CIN due to whole chromosome gain and loss *in vitro* and *in vivo*.[114] When CENP-$E^{+/-}$ transgenic mice were inspected for spontaneous tumor formation, it was found that transgenic animals developed higher rates of lymphomas and lung tumors than their littermates, but significantly decreased rates of tumorigenesis in tissues normally prone to tumor formation, such as the liver. Additionally, CENP-$E^{+/-}$ animals exhibited decreased tumorigenesis compared to wild type animals when exposed to the well characterized carcinogen DMBA and a highly significant increase in tumor-free survival in the absence of the ARF tumor suppressor when compared to $ARF^{-/-}$, CENP-$E^{+/+}$ littermates. Another important observation of the study was that aneuploidy in nontransformed cells *in vivo* was characterized by a disproportionate tendency toward whole-chromosomal loss relative to gain.

Taken together, these results argue that aneuploidy promotes tumorigenesis in otherwise genetically stable tissues and cells but inhibits tumor formation in tissues with a preexisting rate of CIN. This mechanism may explain why cell fusion is tumor suppressive in certain contexts, [74, 125-126] which led to the early belief that tumor cell fusion always suppressed malignancy and even aided in the discovery of tumor suppressor genes.[127]

If high or sustained levels of CIN could result in the extinction of a neoplasia, Storchova et al. raise the possibility that cell fusion via the introduction of additional genetic material gives a sort of "cushion" to the deleterious effects of CIN, as redundant genes could compensate for the loss of single alleles or whole chromosomes.[88] It is also possible that a polyploid genotype after fusion has a larger selection of dormant genes that can be activated in response to environmental challenge and thus display the remarkable adaptive ability found in tumor cells.[48, 51] In any case, in order for a clinically significant tumor to arise, at least one stable genome

capable of continuous proliferation must be generated from the period of CIN (Figure 1A). This seems to be the case in many cancers by the time of clinical appearance,[44, 60, 128] and is further supported by the observation that karyotypes from relapsed tumors many years after treatment can be identical to the original tumor.[129-130]

V. CELL FUSION AND METASTASIS

Although much progress has been made in recent years in understanding the pathways of metastasis and the role of the microenvironment in this process,[131] very little is known about the generation of metastatic cells within a previously non-metastatic primary tumor. The question of why only a tiny fraction of transformed cells are able to free themselves from the cell and ECM adhesions of the tumor site, migrate through the surrounding tissues and basement membranes, intravasate into the bloodstream or lymphatic system, and extravasate, invade, and proliferate at a distant site is a critical one for the development of effective therapeutics. The importance of understanding this process is underscored by the fact that a majority of cancer deaths are attributed not to primary tumors, but to complications arising from distal metastases. A recent publication[132] further emphasizes the importance of understanding metastasis by pointing out that of the six famed "hallmarks of cancer",[133] only one – tissue invasion and metastasis – can be used to distinguish a life-threatening malignant tumor from an essentially harmless benign tumor.

The dogmatic view of metastasis is that it evolves gradually during tumor evolution, as part of the selective adaptation of the tumor genome.[134] In this linear progression model, primary tumor cells accumulate genetic changes randomly, those mutations that support survival and proliferation in the tumor microenvironment are selected for, and clonal expansion occurs

within the tumor. Eventually, a cell arises with a combination of chance genetic alterations that enable it to depart the primary tumor and take up residence elsewhere in the body. This model has become popular for good reason. First, it follows logically from the discovery of oncogenes in malignant transformation and second, it does an excellent job of explaining the positive clinical correlation between tumor size and frequency of metastasis.[134-135] A large tumor has presumably been in existence longer than a smaller tumor, giving it both more time for accumulation of mutations and a larger cell population in which to select for cells capable of metastasis. However, several lines of evidence argue against the linear progression model of metastasis.

First, advances in parallel-sequencing technology now allow for whole-genome analysis of primary and metastatic tumor cells. One recent study compared the whole genome of a brain metastasis to that of the primary breast tumor and found only two *de novo* mutations in the metastatic tumor.[136] One was a silent mutation while the other was determined to be non-essential to metastasis, suggesting that a mechanism other than genetic mutation initiated the metastatic process. It is important to note that this methodology would not detect polyploidy of the tumor cells.

Another argument involves the growth kinetics of primary and metastatic tumors. The linear progression hypothesis predicts that significant time and cell divisions will be required for a tumor cell to gain and "fix" the chance mutations needed for metastasis. Therefore, according to this hypothesis primary tumors should be of sufficient size to sustain the establishment of clonogens that form with low frequency. In fact, metastases are often seen in early disease stages, and 5-10% of patients diagnosed with cancer in Europe and the United States present with unknown primary tumors.[137-138] For the linear progression hypothesis to hold in these cases,

growth rates of metastases would need to be far greater than those of the primary tumor. Primary and metastatic breast cancer growth rates have been well studied[139-140] and argue against this possibility. Several methods of investigation have shown that growth rates of primary and metastatic tumors are comparable at the time of diagnosis, typically within a factor of two.[134]

A third, primarily hypothetical argument against linear progression has been put forward by George Parris[141] and expands on the discussion of CIN and consequent genomic stabilization above. If a tumor is conceptualized as a new, "parasitic" species within the host,[142] it will be subject to the ecological pressures of any other species. By the widely known ecological concept of Muller's Ratchet,[143] an asexual population with only genetic mutations available to generate diversity may gain a momentary genetic advantage via a desirable mutation. However, when a deleterious mutation occurs in this asexual population it must (with the exception of back mutations) be passed on to all progeny. As a given mutation is more likely to be deleterious than advantageous, undesirable mutations build up in an asexual population until it finally becomes extinct. In the context of cancer progression, this theory would allow for the formation of neoplasia due to somatic mutations, but tumors would be expected to become extinct before clinical relevance or metastasis. We and others[13, 74, 141] argue that a "sexual" method of reproduction – such as cell fusion – that allows for genetic recombination offers a more likely explanation for tumor progression into metastasis than the linear progression model (Figure 1B). This hypothesis is initially supported by the correlation between the most immediate consequence of genetic recombination – aneuploidy – and poor prognosis in a variety of cancers[92, 144-147] as well as the finding that polyploidy is strongly correlated with growth rate in many species[148]. In more concentrated studies, it was found that polyploidy in the liver was increased in faster growing mouse pups[149] and in rat hepatocytes after growth hormone

stimulation.[150] Thus, fusion-induced polyploidy may be an adaptive response to the increased rate of proliferation throughout disease progression.

A. Tumor cell fusion with other tumor cells

If fusion is a viable means by which metastasis is initiated, the next question is "which two (or more) cells fuse?" The definitive answer to this question remains elusive, but several studies are suggestive of certain pairings. One pairing is fusion between tumor cells. If tumor cells fuse with each other, detection of such an event within a single tumor is challenging, as most detection techniques rely on overlapping lineage-specific markers to identify fusion partners. Accordingly, a fusion event between phenotypically identical tumor cells would be undetectable in most experimental systems. However, spontaneous fusion between cancer cells has been observed *in vitro*. As early as 1984, it was observed that multi-nucleated giant cells arose in suspension cultures of B16 melanoma cells.[151] More recently, co-culture of two variants of the MDA-MB-231 breast cancer line, one of which metastasizes almost exclusively to bone and the other primarily to the lung, resulted in fusion hybrids that had gene expression signatures of both parental strains and retained strong metastatic ability to both lung and bone *in vivo*.[152] Interestingly, fusion products were phenotypically and genomically stable at a nearly doubled genome size after passage *in vivo* up to the time of publication (almost 1 year).

B. Tumor cell fusion with local non-malignant cells

Evidence of fusion between xeno- or allogeneic transplanted malignant cells and host cells *in vivo* was reported repeatedly in the 1970s and early 1980s,[153-154] often resulting in increased metastatic ability. However, it was not until recently that advances in genetic profiling

techniques such as fluorescence *in situ* hybridization (FISH) could provide conclusive evidence of synkaryon formation.

In the last decade, fusion of epithelial tumor cells with local endothelial and other stromal cells *in vivo* has been reported. In one study, human breast cancer cells were injected into the tail vein of nude mice. Subsequent analysis of lung sections revealed that 0.5-2.0% of tumor cell nuclei contained both human and murine DNA.[155] A portion of these hybrid cells also stained positively for the endothelial marker von Willebrand factor, suggesting that at least some of the murine fusion partners were of endothelial origin. FISH analysis of the hybrid cells revealed consistent spatial separation of human and murine DNA into distinct subcompartments of hybrid nuclei, a phenotype that was reproduced *in vitro*. Although the relevance of this spatial separation is unknown, it is interesting to speculate on the epigenetic and mitotic consequences of this arrangement. It is possible that chromosomes of one fusion partner are epigenetically favored over the other, resulting in transcriptional profiles very similar to parent cells. Alternatively, this compartmentalization could allow for lineage-specific DNA content to be easily separated in subsequent asymmetric cell divisions,[37] resulting in progeny with little or no genomic alterations. Because of the great similarities between the progeny of fusion products and unfused cells either of these mechanisms could lead to underestimation of the frequency of cell fusion by common detection techniques

Further evidence for tumor cell-stromal cell fusion comes from implantation of primary human breast cancer cells from a pleural effusion into the mammary glands of nude mice.[156] Resultant tumor nuclei stained positive for both human and murine DNA. In addition, a cell line was created from the tumor that had a spindle-shaped stromal morphology and stained positive for several stromal markers. After immortalization, FISH analysis indicated that ~64% of

interphase nuclei were mouse-human hybrids, while a significant portion of mitotic cells showed mouse/human chromosomal translocations. The DNA content of the analyzed synkaryons was ~4N in early passages, but gradual reduction of chromosomes was observed upon serial passaging. These data suggest that increased chromosomal content was beneficial for tumor cells adapting to a new environment *in vitro*. Gradual loss of chromosomes with passage number could represent the stabilization of a new karyotype after cell fusion-induced CIN or simply the reduction of genetic load of cultured cells by loss of DNA content unnecessary for survival. Similar behavior is often observed in antibiotic resistant bacterial and mammalian cell lines upon removal of the selective pressure of antibiotics in the culture media.[157]

A more recent study investigated long-term gene expression of hybrid cells formed after transplantation of a primary human glioblastoma into a hamster cheek pouch.[158] After a year of passage *in vivo*, metastases were found to contain primarily hamster DNA, but still retained genes from at least six different human chromosomes as detected by PCR. Additionally, histochemical examination showed protein expression of at least three human genes after one year of passage. All three of the human proteins detected (CD74, CXCR4, and PLAGL2) have been implicated in cancer progression,[158] again suggesting selective retention and/or expression of genes beneficial for tumor cell survival and proliferation after cell fusion.

C. Tumor cell fusion with BMDCs

Perhaps the longest[159] and most extensively investigated tumor cell fusion partners have been bone-marrow derived cells, especially immune cells such as macrophages. This is in part because the consequences of tumor cell fusion with immune cells agree well with numerous observations of cancer progression.

Tumors have been described as "wounds that do not heal"[160] and as such, recruit immune cells through the activation of the body's natural inflammation response. A plethora of BMDCs are known to be present in the tumor microenvironment (reviewed in[161]) and many, including $CD4^+$ T cells[162], mast cells[163], B cells[164], and macrophages[165] have been implicated in cancer progression or poor prognosis. Interestingly, chronic inflammation or other tissue injury has been shown to induce BMDC fusion in a wide variety of tissues, both in a cancer context[53, 166] and in normal regenerative processes[38-39, 167-168]. Especially interesting is the observation that BMDC fusion with diseased hepatocytes results in fusion products that repopulate the liver due to a selective advantage in the microenvironment[19]– a model that could be recapitulated in the tumor microenvironment.

Given these observations, it is not surprising that all three clinical case studies implicating cancer cell fusion have suggested BMDCs as the fusion partner. In two studies reported by Pawelek and colleagues, patients with a prior bone marrow transplant developed renal cell carcinomas.[20, 169] In both cases, marrow-donor DNA was detected in mononuclear tumor cells. In the most striking example, the donor Y-chromosome was identified via FISH analysis in mononuclear tumor nuclei also containing three copies of the female host's 17^{th} chromosome, a signature of the tumor. Importantly, the Y chromosome was detected in a small area at the border of the tumor accounting for ~10% of the tumor area. This observation suggests a possible clonal expansion from the initial fusion event. In a third case, FISH analysis indicated that up to 48% of nuclei in multinucleated osteoclasts of a multiple myeloma patient were of myeloma origin, with all nuclei maintaining activation of transcription.[170]

It has also been suggested that carcinoma cells can undergo an epithelial-to-mesenchymal conversion during the transition from benign to malignant tumors. During this transition, cells

lose tight cell-cell contacts and E-cadherin expression, and gain migratory and invasive capacities of mesenchymal cells (reviewed in [171]). This change in phenotype can be easily imagined as a result of fusion of a tumor cell with an immune cell that depends on efficient migration to function effectively. Indeed, melanoma cell/macrophage fusion products have shown increased migratory capacity *in vitro*[172] and greater metastatic potential *in vivo*[173] compared to the parent melanoma line.

Macrophages are an especially promising fusion partner candidate, as they routinely fuse *in vivo* to form osteoclasts and giant cells.[69] It is worth noting that macrophages also share many characteristic properties of tumors, such as angiogenesis, protease secretion, and growth factor and other cytokine production.[174] Pollard and colleagues have pioneered the study of tumor-associated macrophages (TAMs) in mice models and have suggested that TAMs are essential for metastasis.[175] In human patients, macrophage density around the primary tumor has been correlated to poor prognosis in several studies.[165, 176-177]

The characteristics of macrophage/melanoma fusion products *in vitro* have been studied extensively by Pawelek and colleagues and have been shown to exhibit multiple metastatic characteristics absent in the parent melanoma line, including increased motility,[172] increased proto-oncogene expression,[178] and macrophage-associated expression of β1,6 oligosaccharides.[179] Notably, many of these characteristics were reproduced after culturing cells from spontaneous metastases developed *in vivo*.[173] In this important study, a nude mouse with a homozygous tyrosinase mutation (c/c) developed a tumor after subcutaneous implantation of wild type (C/C) melanoma cells. Genetic analysis of metastases revealed a C/c genotype and a 30-40% increase in DNA content, suggesting the tumor cells had fused with host cells, possibly with metastasis as a direct consequence. Subsequent *in vitro* culture of cells isolated from the

metastases revealed characteristics similar to artificially derived melanoma-macrophage hybrids, such as increased motility and β1,6 oligosaccharide expression.

Additional evidence of *in vivo* tumor cell fusion with macrophages has been supplied recently in a study by Powell et al., in which green fluorescent protein (GFP)-labeled macrophages were introduced via parabiosis into a host harboring β-galactosidase-expressing intestinal tumors.[53] Double-labeled cells were observed in up to 20% of the tumor epithelia after parabiosis, however, as the intestinal epithelium completely renews every 3-5 days[180] it is unclear whether labeled macrophages fused with tumor epithelia or rather with a progenitor cell that later differentiated into epithelium, as has been shown previously.[166] In any case, transcriptome analysis of fusion products revealed transcription profiles with similarities to both native epithelial and macrophage transcriptomes. Significantly, over 3% of differentially regulated transcripts in the fusion products were unique in the fusion product compared to either parental cell line, providing evidence that nuclear reprogramming following cell fusion can result in gene expression completely distinct from either parent cell.

VI. ALTERNATIVE MECHANISMS OF GENE TRANSFER

Although cell fusion offers a promising route for genetic recombination and diversity generation within tumors, it is not the only possible mechanism. Macrophages also routinely perform phagocytosis and digestion of apoptotic cells, a process that has been shown to result in horizontal transfer of oncogenes into the phagocytic host.[181-182] Macrophages are known to associate extensively with necrotic areas in advanced tumors[177] and it is reasonable to speculate that clearance of necrotic debris by macrophages may also result in genetic transfer of tumor cell DNA into the macrophage genome.

Tumor cell secretion of microvesicles via membrane blebbing and other mechanisms has been a recent focus of study.[183] These microvesicles have not been shown to contain DNA, but commonly harbor RNA and proteins, including activated oncogenic proteins.[184] Microvesicles could therefore transfer information between tumor cells or from tumor cells to the stroma through RNA or protein, bypassing the need for genetic recombination. Similarly, membrane nanotubes allow for transfer of vesicles and signaling molecules between cells, although evidence of these structures *in vivo* is lacking.[185]

It is also possible that information flows in the opposite direction, from the stroma to the tumor. A recent model of tumor progression returns to the idea of the tumor as a parasite. The model posits that tumor cells induce autophagy in the stromal microenvironment through oxidative stress, resulting in a release of nutrients that are used by the tumor for continued growth.[186] Although autophagy is normally thought to preserve genomic DNA content, mitochondrial DNA could be transferred to the tumor in this manner. Alternatively, information could again be transferred via RNA or proteins.

Finally, naked DNA is known to be taken up and expressed by a variety of cell types *in vivo*.[187] It is possible that DNA is released into the tumor microenvironment through apoptosis or necrosis and is simply randomly incorporated into the genome of tumor or stromal cells.

VII. DETECTION OF CELL FUSION

Given the potential impact, it is surprising that fundamental aspects of tumor cell fusion are unknown, including the soluble or insoluble signals that trigger fusion, the cell surface proteins responsible for mediating fusion, the kinetics of fusion within the metastatic cascade, or the mechanism of reprogramming and the functional capacity of hybrid cells after fusion. Hindering discovery in this area is lack of appropriate technology to 1) identify fusion partners

poised to fuse and to 2) track fusion products over time. To address this problem we have recently developed imaging and characterization technologies to accurately identify fusion products immediately after cell fusion and to track fusion products over time both in vitro and in vivo.

A. Historical Methods

The origin of a cell as the product of a fusion event can be difficult to deduce since in many cases hybrid cells are morphologically identical to unfused cells (Figure 2B). Three methods have been used traditionally to overcome this difficulty. First, cell fusion can be detected using fluorescent cytoplasmic dyes which diffuse freely through the membranes of live cells. Once inside the cell, these mildly thiol-reactive probes react with intracellular components to produce cells that are fluorescent for at least 24 hours after labeling. Different dyes are used to label each fusion partner and fusion products are discerned by detecting overlapping fluorescence emission via flow cytometry or fluorescence microscopy. This method is ideal for short term *in vitro* studies; however *in vivo* studies are not possible and the fluorescence signal is undetectable at 72 hours in proliferating cells. In addition, dead cells can be endocytosed and the cell dye transferred as a consequence leading to false positives. Second, cell fusion can be detected by the complementary action of genes; most commonly, the Cre-Lox system.[18] This method is robust, detecting only true fusion events and can be used *in vivo*. However, available reporter systems (i.e., beta-galactosidase, fluorescent proteins) limit *in vivo* analysis to excised tissues precluding the tracking of fusion events in the same organism over time. Third, fusion events can be detected by probing the DNA of different species or genders (X, Y chromosomes). When a cell of one species/gender fuses with a cell of another species/gender, *in situ* hybridization probes can be generated to species/gender-specific sites in the genome. More

recently, genetic techniques such as single nucleotide polymorphism (SNP) and short-tandem repeat (STR) analysis have been utilized to identify DNA from multiple parents in fusion products.[188] However, these methods of detection of fusion events are also limited to excised tissue. Thus, there is a distinct lack of means to detect and track fusion products long term *in vitro* and *in vivo*.

B. New Approaches

We have developed two new approaches for the detection of fusion products. The first utilizes bimolecular fluorescence complementation (BiFC). BiFC is a method of viewing the association of proteins inside living cells. Intact GFP (and its variants YFP, BFP, RFP, etc.) is fluorescent, and this property can be reconstituted when these proteins are broken into two halves by making each into an interactive fusion protein (Figure 2A).[189-191] Fluorescence is detected and recorded via traditional, time lapse, fluorescence microscopy. We believe this technique to be a powerful tool for detecting fusion *in vitro* and will be especially useful to determine the mechanism by which the fate of tumor cell hybrids is accomplished (i.e., nuclear fusion, rearrangement of genetic material or other). Due to the inducible nature of the signal (i.e., fluorescent signal is detected only after a fusion event, instead of detection of overlapping fluorescent signals), the incidence of false-positives is essentially zero. In addition, the hybrids synthesize accumulating amounts of BiFC genes over time and so signal intensity increases over time (Figure 2B), instead of degrading over time as is the case for cytoplasmic dyes. However, this approach is limited in that it cannot easily be adapted to *in vivo* systems.

To detect fusion products *in vivo* we have developed an approach to trigger bioluminescence upon fusion. We have developed a construct encoding the firefly (*Photinus*

pyralis) luciferase gene placed downstream of a stop codon flanked by LoxP sequences (Figure 3A). When cells expressing this gene fuse with cells expressing the Cre protein, the LoxP sites are recombined resulting in excision of the stop signal and expression of luciferase (Figure 3B).[192] Akin to the BiFC method, this is an inducible method thereby limiting the incidence of false-positive signals. Unlike existing methods which utilize the Cre/Lox system, we have incorporated a "living" detection signal and thereby afford for the first time the opportunity to track the kinetics of cell fusion *in vivo*.

VIII. CANCER RELEVANCE

Despite mounting evidence that cell fusion or transfer of genetic material by various mechanisms could contribute to carcinogenesis and metastasis, critics contend that spontaneous fusion occurs too rarely to contribute in a relevant way to the initiation and propagation of diseases like cancer. In response, we offer a few points for consideration. First, we point to the lack of technology sensitive enough to determine the relative frequency of spontaneous fusion between tumor cells or their precursors with surrounding cell types. As noted above, the task is complicated by the uncertainty of rearrangement of cytoplasmic and nuclear components and the relative speed with which rearrangement takes place.

The choice of model system will be important to the assessment of the role of fusion in the future. Since it is unlikely that fusion assays will ever be done in humans, rodent models are an obvious choice. However, many mouse models are not spontaneously metastatic. The human tumor cell lines or human grafts that are in common use have already progressed past the point of genetic evolution likely to be catalyzed by cell fusion, and results from these may not reveal the full potential of this mechanism. Even if early stage lesions were transplanted, the mouse

hosts will be immune-compromised, and this microenvironment may therefore be deficient in key cell fusion partners. It will be important to choose the correct assay to test this hypothesis.

IX. CONCLUSION

We have discussed the processes that regulate cell fusion, as it occurs naturally and spontaneously to govern normal tissue development and functionality, and the evidence for novel cell fusion events (and/or genetic transfer) that occur during pathogenic processes. These include viral infections, which include fusion events at various points of their life cycles, and the immune reactions that characterize inflammation. The details of these processes are known, including their molecular mediators. Given the emerging importance of inflammatory mediators during metastasis, it seems a logical extension to propose that cell fusion will be associated with this process, and that it could indeed be a functional effector of neoplastic changes. Cell fusion offers an opportunity for rapid genomic evolution. It may offer practical solutions to explain the (arguably) paradoxical timelines for tumors, and to generate mechanisms for the evolution of the massively rearranged genomes that are so characteristic of these tumors. Cell fusion can account for re-assortment of combinations of mutant and normal genomes in various copy numbers. It also offers the potential to acquire cytoplasmic determinants of function (including mitochondria, trafficking components and cell signaling-related functions) that have been shown historically to be powerful mediators of the cancer cell phenotype. These functionalities may be acquired only transiently, to enable for example the enhanced motility or altered metabolism required for metastasis, and may largely revert after the fusion products resolve to stable daughter cells in metastatic outgrowths. The potential of this novel hypothesis stands on the brink of a full evaluation, with the development of new tools and methods. If these studies generate evidence

in support of this hypothesis, this avenue opens the way to a whole new class of therapeutics that are likely to be highly selective for pathogenic processes.

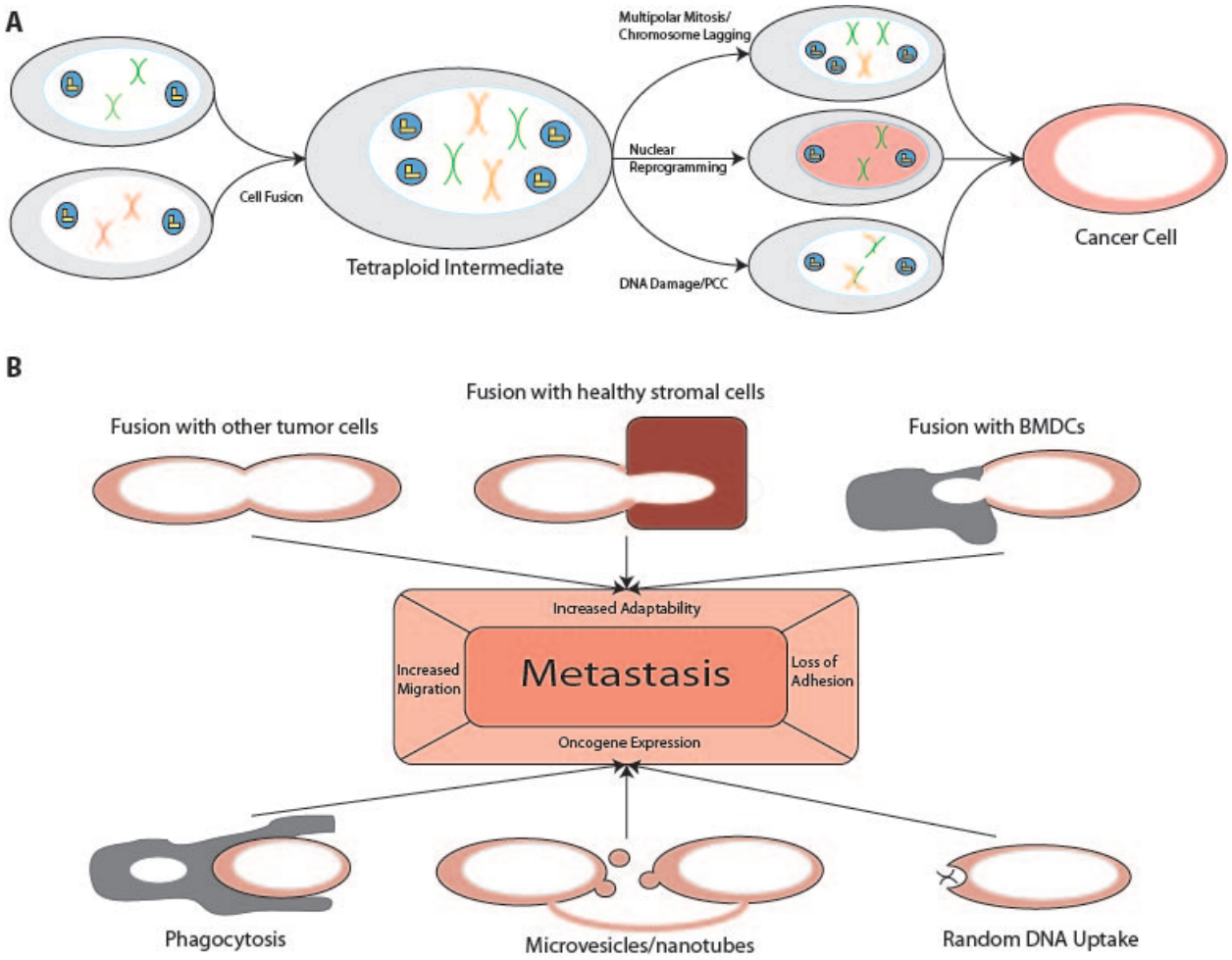

Figure 1. Schematic of cancer progression via cell fusion. A) Fusion can lead to oncogenesis. An initial fusion event leads to an intermediate tetraploid fusion product with doubled centrosome content. This intermediate is unstable and is capable of progression into malignancy through several mechanisms. **B)** Fusion can lead to metastasis. Cell fusion or alternative methods of genetic transfer impart new phenotypic capabilities to the tumor cell which enable the cell to metastasize.

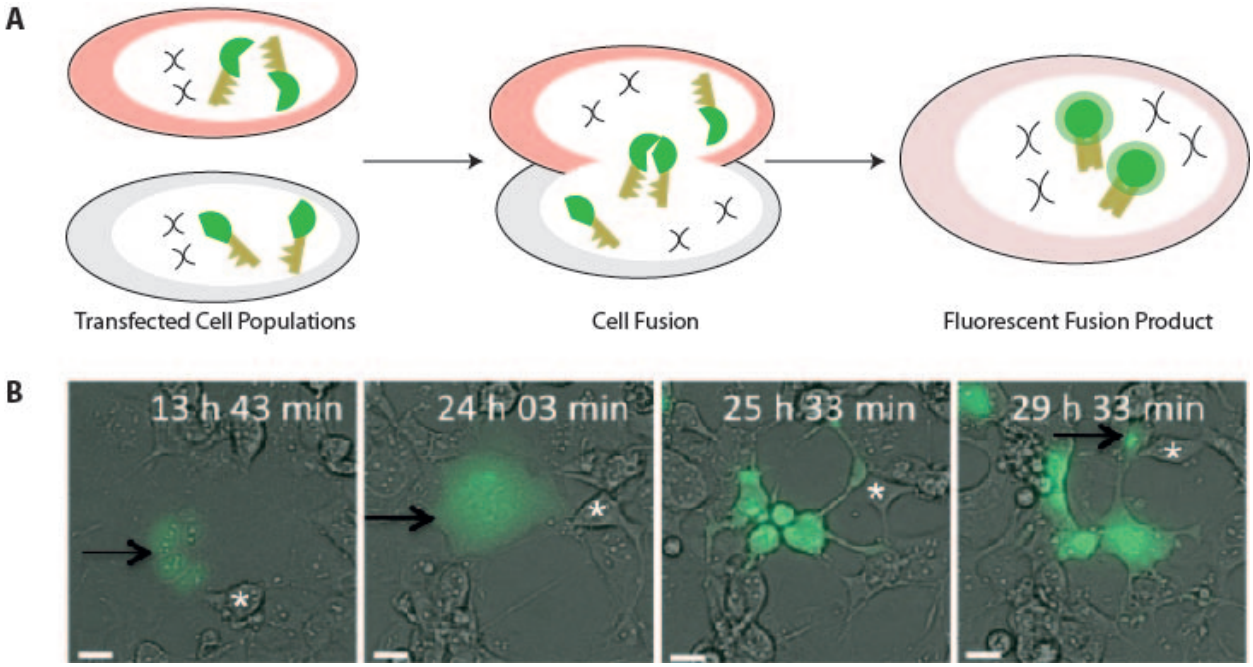

Figure 2. Detection of cell fusion *in vitro* via BiFC. A) Schematic of BiFC mechanism. Each BiFC construct contains half a YFP gene fused to genes encoding histone H3.1, which naturally dimerizes in the cell. The two BiFC constructs are transduced into separate cells. When the cells fuse, the YFP protein is reconstituted and a fluorescent signal is generated. **B)** Morphologic comparison of fusion products with unfused cells.[191] Plasmids corresponding to complementary BiFC constructs (YN-CBX5 and YC-CBX5) were transfected into separate populations of COS-1 cells. After transfection, populations were mixed and fusion was induced with poly(ethylene glycol). Fusion signals (green) were detected using fluorescence microscopy. The morphology of fused cells was monitored via time-lapse imaging for 30 hours. The initial fusion product is a clearly larger cell (black arrow, first two frames). When this cell divides, the four daughters remain green (and would be difficult to distinguish from other non-fused cells (white asterisk) were it not for the BiFC label).

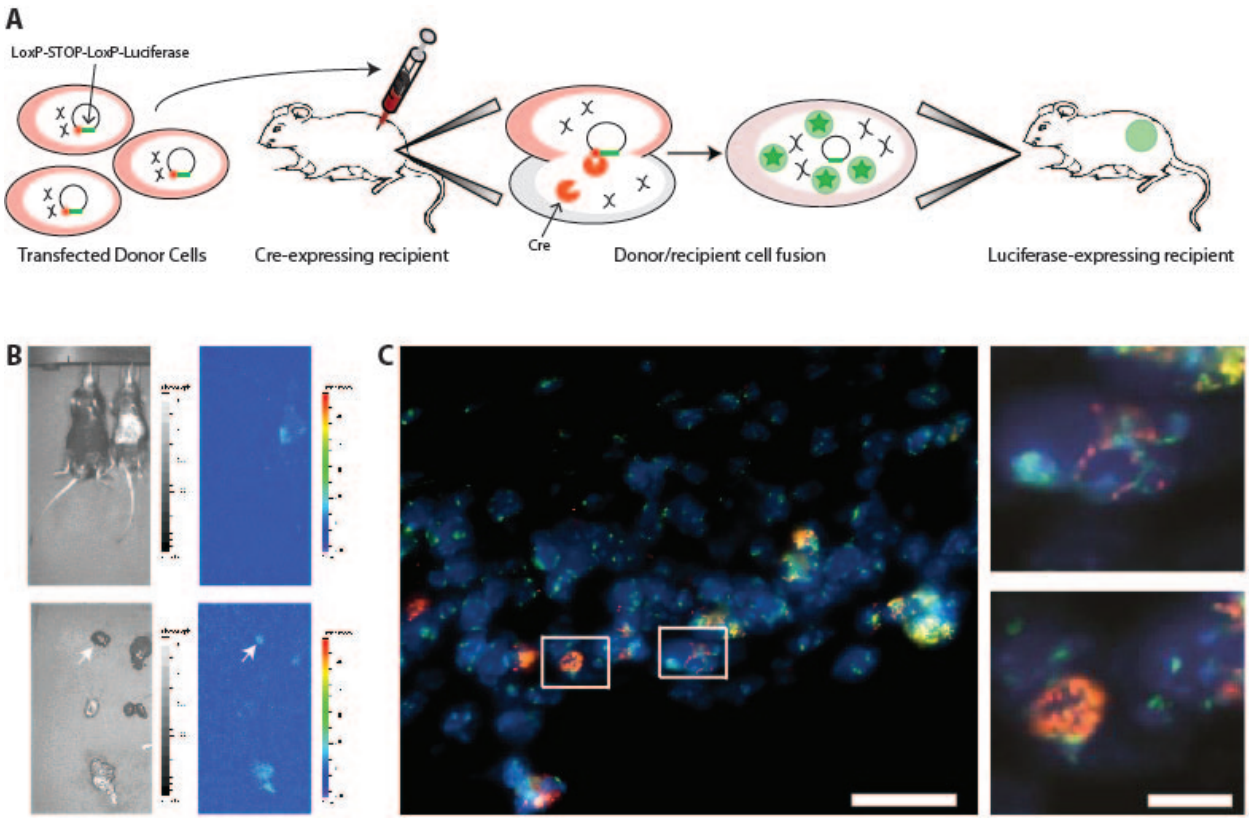

Figure 3. **Detection of cell fusion *in vivo* via Cre-LoxP bioluminescence. A)** Schematic of Cre-LoxP bioluminescence mechanism. Upon donor/recipient cell fusion, the Cre protein excises the floxed stop codon and luciferase is expressed in the fusion product. **B)** Detection of cell fusion *in vivo* via bioluminescent imaging. Human MSCs were transfected with two plasmids, one containing luciferace downstream of a floxed stop codon and the other containing the viral fusogen VSVG. Transfected MSCs were then transplanted into Cre-expressing mice. Luminescent signal was present in experimental mice (top, right mouse) but not in sham controls (top, left mouse). Excision of organs (bottom) revealed luminescent signal, indicative of fusion, in multiple tissues including the heart (arrow). **C)** FISH analysis of bioluminescent heart tissue.[193] Insets display fusion products with nuclei staining positive for both human centromeres (red) and mouse centromeres (green). One fusion product (bottom right) is undergoing mitosis. Scale bar = 25μm.

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